



Enabling Discovery in Life Science®

Mito-ID® Green Detection Kit

for fluorescence microscopy

Instruction Manual

Cat. No. ENZ-51022-K500 500 assays

For research use only.

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Notice to Purchaser

The Mito-ID® Green Detection Kit is a member of the CELLestial® product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial® reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

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I. Introduction

Enzo Life Sciences' Mito-ID[®] Green Detection Kit contains a novel mitochondria-selective dye suitable for live-, detergent-permeabilized and aldehyde-fixed cell staining. Conventional fluorescent stains for mitochondria, such as JC-1 (Cat. No. ENZ-52304), rhodamine 123 (Cat. No. ENZ-52307 and tetramethylrhodamine ethyl ester (TMRE, Cat. No. ENZ-52309), are readily sequestered by actively respiring mitochondria. However, these mitochondrial stains are subsequently leached out of cells once the mitochondria's membrane potential dissipates. This characteristic severely limits their use in experiments in which cells must be treated with nonionic detergents, aldehyde fixatives or other agents that affect the energetic state of the mitochondria. Micromolar concentrations of Mito-ID[®] Green dye are sufficient for staining mammalian cells. This has been validated with a human cervical carcinoma cell line, HeLa, a human T-lymphocyte cell line, Jurkat, and human bone osteosarcoma epithelial cell line, U2OS.

One important application of Mito-ID[®] Green dye is in fluorescence co-localization imaging with red fluorescent protein (RFP)-tagged proteins. This is a powerful approach for determining the targeting of molecules to intracellular compartments, and for screening of associations and interactions between these molecules. Additionally, many organelle-targeting probes photobleach rapidly, are subject to quenching when concentrated in organelles, are highly toxic, or only transiently associate with the target organelle, requiring imaging within a minute or two of dye addition.^{3,4}

Enzo's Mito-ID[®] Green dye, a cell-permeable small organic probe molecule that spontaneously localizes to live or fixed mitochondria, was developed to overcome the above problems. The Mito-ID[®] Green dye can be readily used in combination with other common UV and visible light excitable fluorescent dyes and various fluorescent proteins in multi-color imaging and detection applications. It emits in the FITC region of the visible light spectrum, and is highly resistant to photobleaching and concentration quenching.

The Mito-ID[®] Green Detection Kit has been specifically designed for use with RFP-expressing cell lines, as well as cells expressing blue or cyan fluorescent proteins (BFPs and CFPs). Additionally, the kit is suitable for use with live or fixed cells in conjunction with fluorescent probes, such as labeled antibodies, or other fluorescent conjugates displaying similar spectral properties as coumarin, Texas Red and cyanine 5. A nuclear counterstain, Hoechst 33342, is provided to highlight this organelle as well.

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at $\leq 20^{\circ}\text{C}$, protected from light. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for approximately 500 assays using either live, adherent cells or cells in suspension.

Reagent	Quantity
Mito-ID [®] Green Detection Reagent	50 μL
Hoechst 33342 Nuclear Stain	50 μL
10X Assay Buffer	15 mL

III. Additional Materials Required

- Standard fluorescence microscope
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Glass microscope slides
- Glass cover slips
- Deionized water
- Anhydrous DMSO (optional)
- Growth medium (e.g., Dulbecco's Modified Eagle Medium, D-MEM)
- Formaldehyde (optional, for fixation protocol)
- Triton X-100 (optional, for permeabilization protocol)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- The Mito-ID[®] Green Detection Reagent contains DMSO which is readily absorbed through the skin. It is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

V. Methods and Procedures

NOTE: *Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

A. REAGENT PREPARATION

1. 1X Assay Buffer

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (mL) of the 10X Assay Buffer with 9 mL of deionized water.

2. Dual Detection Reagent

The concentration of Mito-ID[®] Green dye for optimal staining will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed and other factors such as the permeability of the dye to the cells or tissues. To reduce potential artifacts from overloading of the cells, the concentration of the dye should be kept as low as possible.

Prepare sufficient amount of Dual Detection Reagent for the number of samples to be assayed as follows: For every 1 mL of 1X Assay Buffer (see preparation in step 1) or cell culture medium, add 1 μ L of Mito-ID[®] Green Detection Reagent and 1 μ L of Hoechst 33342 Nuclear Stain.

- NOTE:** (a) *The dyes may be combined into one staining solution or each may be used separately, if desired.*
- (b) *The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than the green Mitochondrial stain, Mito-ID[®] Green.*
- (c) *When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.*

B. STAINING LIVE, ADHERENT CELLS

1. Grow cells on cover slips inside a Petri dish filled with the appropriate culture medium. When the cells have reached the desired level of confluence, carefully remove the medium.
2. Dispense sufficient volume of Dual Detection Reagent (see section V-A2, page 3) to cover the monolayer cells (~100 μ L of labeling solution for cells grown on an 18 X 18 mm coverslip).
3. Protect samples from light and incubate for 15-30 minutes at 37°C.
4. (Optional) Wash the cells with 100 μ L 1X Assay Buffer. Remove excess buffer and place coverslip on slide.
5. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard FITC filter set for imaging the mitochondria. Optionally, image the nucleus using a DAPI filter set and the RFP-tagged protein using a Texas Red filter set.

C. STAINING LIVE CELLS GROWN IN SUSPENSION

1. Centrifuge cells for 5 minutes at 400 x *g* at room temperature (RT) to obtain a cell pellet.
2. Carefully remove the supernatant by aspiration and dispense sufficient volume of Dual Detection Reagent (see section V-A2, page 3) to cover the dispersed cell pellet.
3. Protect samples from light and incubate for 15 to 30 minutes at 37°C.
4. (Optional) Wash the cells with 100 μ L 1X Assay Buffer. Remove excess buffer. Resuspend cells in 30 μ L 1X Assay Buffer, then apply the cells to a glass slide and overlay with a coverslip.
5. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard FITC filter set for imaging the mitochondria. Optionally, image the nucleus using a DAPI filter set and the RFP-tagged protein using a Texas Red filter set.

D. STAINING OF ALDEHYDE-FIXED AND DETERGENT PERMEABILIZED CELLS

The Mito-ID® Green dye is capable of staining already fixed and permeabilized cells.

1. Growth of cells should be performed as described in sections B or C.
2. Carefully remove the growth medium, or 1X Assay Buffer covering the cells, and replace it with freshly prepared medium or buffer containing 3.7% formaldehyde.
3. Incubate the cells at 37°C for 15 minutes.
4. After fixation, wash the cells in PBS or 1X Assay Buffer.
5. If the cells are to be subsequently labeled with an antibody, a permeabilization step is usually required to enhance the antigen's accessibility. Incubate the fixed cells in PBS or 1X Assay Buffer containing 0.1% Triton X-100 at room temperature for about one minute.
6. Following permeabilization, rinse the cells twice in PBS or 1X Assay Buffer.
7. Perform staining as recommended for adherent or suspension cells (sections B or C) using a 500-fold dilution of the Mito-ID® Green instead of the 1000-fold dilution.

NOTE: *If desired, standard immunofluorescence staining protocols using coumarin-based, cyanine 5 or other red or blue fluorescent antibody conjugates, or equivalent, should be performed before Staining with Mito-ID® Green. Antifade compounds or mounting media may be beneficial.*

Try to view the samples as soon as possible after staining for sharper staining.

VI. APPENDICES

A. Filter Set Selection

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope.

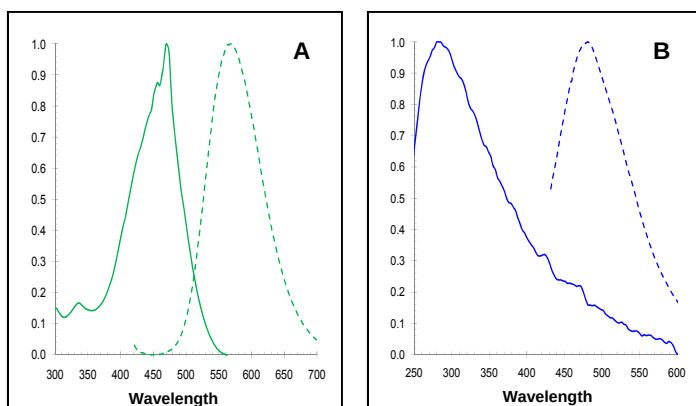


Figure 1. Absorption/excitation and fluorescence emission spectra for Mito-ID[®] Green [Ex/Em: 460/560 nm] (panel A) and Hoechst 33342 [Ex/Em: 350/461 nm] (panel B) dyes. All spectra were determined in 1X Assay Buffer.

B. Results

Mitochondria are subcellular organelles found in eukaryotic cells, often representing as much as 10% of the total cell volume. Although conventional fluorescent stains for mitochondria, such as JC-1, rhodamine 123 and tetramethylrhodamine, are readily sequestered by functioning mitochondria, they are subsequently leached out of the cells once the mitochondrial membrane potential is dissipated. Mito-ID[®] Green dye accumulates in the mitochondria regardless of the mitochondrial membrane potential. The dye selectively stains mitochondria of living cells and is relatively insensitive to mitochondrial membrane potential uncouplers of phosphorylation, such as CCCP (carbonyl cyanide 3-chlorophenylhydrazone), as well as ion-channel drugs, such as valinomycin. In addition to being a live cell-permeable dye, the Mito-ID[®] Green dye is functional after cell fixation and detergent permeabilization.

Mito-ID[®] Green dye has been shown to co-localize with Mito-ID[®] Red dye. Previously, the Mito-ID[®] Red dye has been shown to co-localize with mitochondria expressing a mitochondrial-GFP tag.

VII. References

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2. Nadrigny, Li, Kemnitz, Ropert, Koulakoff, Rudolph, Vitali, Giaume, Kirchhoff and Oheim (2007) "Systematic colocalization errors between acridine orange and EGFP in astrocyte vesicular organelles" *Biophys J.* 93(3):969-980.
3. Minamikawa, Sriratana, Williams, Bowser, Hill and Nagley (1999) Chloromethyl-X-rosamine (MitoTracker Red) photosensitises mitochondria and induces apoptosis in intact human cells. *Journal of Cell Science* 112, 2419-2430.
4. Scorrano, Petronilli, Colonna, Di Lisa and Bernardi (1999) Chloromethyltetramethylrosamine (Mitotracker Orange™) Induces the Mitochondrial Permeability Transition and Inhibits Respiratory Complex I: Implications for the mechanism of cytochrome c release" *J. Biol Chem.* 274, 35, 24657–24663.

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Mitochondria are not sufficiently stained.	Very low concentration of Mito-ID™ green dye was used or dye was incubated with the cells for an insufficient length of time.	Either increase the labeling concentration or increase the time allowed for the dye to accumulate in the mitochondria.
Precipitate is seen in the 10X Assay Buffer.	Precipitate forms at low temperatures.	Allow solution to warm to room temperature or 37°C, then vortex to dissolve all precipitate.
Blue nuclear counterstain is too bright compared to the green mitochondrial stain.	Different microscopes, cameras and filters may make some signals appear very bright.	Reduce the concentration of the nuclear counterstain or shorten the exposure time.
Cells do not appear healthy	Some cells require serum to remain healthy.	Add serum to stain and wash solutions. Serum does not affect staining. Normal amounts of serum added range from 2% to 10%.



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